Physiology and biochemistry of primary alcohol oxidation in the gram-positive bacteria "amycolatopsis methanolica" and "bacillus methanolicus"

Hektor, Harm Jan

Chapter 6

Identification of a new NAD(P)(H)-binding domain in the nicotinoprotein methanol dehydrogenase from *Bacillus methanolicus*

H.J. Hektor and L. Dijkhuizen

submitted to European Journal of Biochemistry

Summary

The *Bacillus methanolicus* methanol dehydrogenase (MDH) has been identified as a member of Family III of NAD(P)-dependent alcohol dehydrogenases (ADHs); it is a decameric nicotinoprotein with one Zn^{2+}-ion and 1-2 Mg^{2+}-ions plus a tightly bound cofactor NAD(H) per subunit. Its relatively low coenzyme NAD-dependent MDH activity is strongly stimulated in the presence of a *B. methanolicus* activator protein. Biochemical studies have shown that activator protein facilitates re-oxidation of the NADH cofactor during enzyme catalysis.

Members of Family III of NAD(P)-dependent ADHs contain three unique conserved sequence motifs (A, B and C). The domain with motif C is thought to be involved in metal-binding, while the functions of domains A and B are still unknown. A classical βαβ-binding fold for NAD(P)(H) is absent in MDH and most other Family III ADHs. The domain A motif contains a βαβ-binding fold and shows some resemblance with the FAD-binding domain of alcohol oxidase. Site-directed mutagenesis of MDH allowed identification of amino acid residues in motif A involved in NAD(H)-binding (G95, S97, D100, K103). Mutants D100N and K103R were completely inactive and had lost cofactor NAD(H). Mutants S97G and G95A were still active but also had lost cofactor NAD(H). Compared to wild type MDH, mutant S97G had an extremely high coenzyme NAD-dependent MDH activity; the less active mutant G95A displayed a linear relation between methanol concentration and specific activity up to 2.5 M methanol. Neither of these four mutant MDHs could be activated by the activator protein anymore.

Introduction

Methanol dehydrogenase (MDH) of *Bacillus methanolicus* belongs to Family III of NAD(P)-dependent alcohol dehydrogenases (ADHs)(Vonck *et al.*, 1991; De Vries *et al.*, 1992), distinct from the long-chain, zinc-containing (Family I) and short-chain, zinc-lacking (Family II) ADHs (Jörnvall *et al.*, 1987; Reid and Fewson, 1994). The initial members of Family III all were iron-dependent ADHs. With an increasing number of member proteins being characterized, it became clear that not all members were iron-dependent. Where investigated, other metals like zinc and magnesium also were found instead of iron (Reid and Fewson, 1994). Identification of ADH members of Family III thus increasingly became based on overall sequence similarity. Three unique conserved sequence motifs have been defined for this family, aiding in ADH classification (Bairoch, 1992; De Vries *et al.*, 1992; Chapter 4) (Table 1). Database searches identified a total of 24 fully sequenced members of Family III containing these conserved motifs (Chapter 4). Several of these are

Figure 1. Partial alignment of Family III ADHs. A. Optimal alignment with the NAD-binding consensus sequence proposed by Wierenga *et al* (1986). B. Alignment with motif A (Table 1). For abbreviations see table 2, Chapter 4 (page 64).

A B

```
AEHBDH         ------------------MAFIYYLTHIHLDFGAVSLLKS---ECERIGIRRP--LLVTDKGVVA  42  ...   74  GCDGLVAVGGGSSIDLAKGIAILA  98
AETDLI         ----------------MKKFTLDYLSPRVVFGAGTASAL-PDEIGRLGARRPLVLSSPEQRELA  47  ...   85  DADSIIAIGGGSTTGLAKILSMNL  109
AMMNO          ---------MQVDELLKPFPIKEFHPFPRALLGPGAHEMI-GPEALKLG--FKKTLVMTSGLRGS  53  ...   96  KCDSFVSIGGGSSHDACKGARISV  120
BCMAR          ----------------MNAFLFEARIPRVVFGAGALQHL-VREIDAMGSTRALVLSTPEQSADA  47  ...   85  GADCAVAIGGGSTTGLGKAIALES  109
BMMDH          --------------------MTNFFIPPASVIGRGAVKEV-GTRLKQIG--AKKALIVTDAFLHS  42  ...   86  NCDALVSIGGGSSHDTAKAIGLVA  110
CAADH          --------------------MMRFTLPRDIYYGKGSLEQ--LKNLKGKK--AMLVLGGGSMKRFG  41  ...   83  EPDWIIAMGGGSPIDAAKAMWIFY  107
CAAADC   448   -------------------MLWFRVPHKVYFKFGCLQFA-LKDLKDLK--KKRAFIVTDSDPYN  490 ...  534  MPDTIIALGGTPEMSSAKLMWVLY  558
CABDHI         ----------------MLSFDYSIPTKVFFGKGKIDVI-GEEIKKYG----SRVLIVYGGGSI  42  ...   88  NVDLVLAIGGGSAIDCSKVIAAGV  112
CABDHII        ----------------MVDFEYSIPTRIFFGKDKINVL-GRELKKYG----SKVLIVYGGGSI  42  ...   88  GVEVVLAIGGGSAIDCAKVIAAAC  112
CFDHAT         ---------------MSYRMFDYLVPNVNFFGPNAISVV-GERCKLLG--GKKALLVTDKGLRA  46  ...   92  HCDIIVTVGGGSPHDCGKGIGIAA  116
CKHBDH         -------MKLLKLAPDVYKFDTAEEFMKYFKVGKGDF-ILTNEFLYKP--FLEKFNDGADAVFQE  55  ...   78  QYNRIIAVGGGSVIDIAKILSLKY  102
HDVADH         --------------AVREQVYGFFIPSVTLIGIGAAK-AIPEKIKALG--GSKPLIVT        41
DGADH          --------------AVREQVYGFFIPSVTLIGIGASK-EIGDKIRRLG--HKFALVIND       42
EC387          ------------------MNNFNLHTPTRILFGKGAIAG--LREQIPHDA---RVLITYGGGSVK  42  ...   85  KVTFLLAVGGGSVLDGTKFIAAAA  109
ECADHC   451    --------------------MLWHKLPKSIYFRRGSLPIA-LDEVITDGH--KRALIVTDRFLFN  493 ...  537  KPDVIIALGGGSPMDAAKIMWVMY  561
ECPOR          ------------------MMANRMILNETAWFGRGAVGAL-TDEVKRRGY--QKALIVTDKTLVQ  44  ...   88  GADYLIAIGGGSPQDTCKAIGIIS  112
ECYIAY         ------------------MAASTFFIPSVNVIGADSLTDA-MNMMADYG--FTRTLIVTDNMLTK  44  ...   88  NCDSVISLGGGSPHDCAKGIALVA  112
EHADH    458   --------------ADRRNNLQWFRVPPKIFFEPHSIRY--LRELKELS----KIFIVSDRMMYK  503 ...  549  GPDNIIAIGGGSAMDAAKIMRLLY  573
EHPADH         ---------------MTMLNFTYYNPVRLIYGKGSLDEIEKQHLIPEDA---RIMMTYGGGSIK  46  ...   87  KINFLVAVGGGSIIDATKYIALGM  111
KPPOR          ---------------MSYRMFDYLVPNVNFFGPNAISVV-GERCQLLGG--KKALLVTDKGLRA  46  ...   92  QCDIIVTVGGGSPHDCGKGIGIAA  116
MGMNO          ---------AIELNQIWDFPIKEFHPFPRALLGVGAWDIAGVL-AKNLGF--KDTLLMGD      48
PSTDLI         ------------------MNFIHDPLTPRVLFGAGRLQSL-GEELKLLGI--RRVLVISTPEQRE  44  ...   84  GVDSYVAPGGGSTIGLAKMLALHS  108
RHTHCE         -------MAIELNQIWD-FPIKEFHPFPRALMGVGAHDIIGVE-AKNLGF--KRTLLMTTGLRGS  54  ...   97  KCDSIISIGGGSSHDAAKGARVVI  121
SCADH4         ----------------MSSVTGFYIPPISFFGEGALEET-ADYIKNKDY--KKALIVTDPGIAA  45  ...   89  NSEIVVSIGGGSAHDNAKAIALLA  113
SPADH     26   CNQSFTNGLKHQSTSSKAMPVSAFYIPSFNLFGKGCLAEA-AKQIKMSGF--KNTLIVTDPGIIK  88  ...  131  NCDSMVSIGGGSAHDCAKGIALLA  155
STEUP          MQAELQTALFQAFDTLNLQRVKTFSVPPVTLCGLGALGACGQEAQAR-GV--SHLFVMVDSFLHQ  62  ...  106  ACDGVVAFGGGSVLDAAKAVALLV  130
ZMADHII        ------------------MASSTFYIPFVNEMGEGSLEKA-IKDLNGSGF--KNALIVSDAFMNK  44  ...   88  NSDFVISLGGGSPHDCAKAIALVA  112
                                        @# # G G  G   #  #          # # D
                                                                      E
                                                                                    .. **..     *
                                                                                    VA GGGS  D  K
```

@: Basic or hydrophilic (K, R, H, S, T, Q, N)
#: Small and hydrophobic (A, I, L, V, M, C)

Table 1. Conserved amino acid sequence motifs for Family III NAD(P)-dependent alcohol dehydrogenases (Chapter 4). Positions correspond to the MDH sequence.

	Motif[a]	Sequence
A	91-103	$V^1\,A^1\,X\,G\,G\,G^1\,S^1\,X_2\,D^2\,X_2\,K$
B	158-199	$K\,X_9\,P^2\,X_5\,D^2\,P^1\,X_6\,P\,X_9\,D^2\,X_3\,H^2\,X_2\,E$
C	258-290	$G^2\,X_2\,H\,X\,M^1\,X\,H^2\,X_2\,G^1\,G^1\,X_5\,H^2\,G^1\,X_3\,A^2\,X_3\,P^2\,X_5\,N^2$

X indicates a non-discriminating position. [1] Similar amino acids are allowed, according to the scheme: PAGST, DE, KR, QN, ILMV, FWY, H and C. [2] Conserved in more than 80 % of the 24 members of Family III.

putative proteins, with only the gene sequences known and limited and no biochemical data available.

Purification of five members of Family III ADHs revealed that each of these proteins possesses a decameric quaternary structure: MDH of *B. methanolicus*, methanol:NDMA oxidoreductase (MNO) of *Amycolatopsis methanolica*, MNO of *Mycobacterium gastri* MB19, ADH of *Desulfovibrio gigas* and ADH of *Desulfovibrio* HDv (Arfman *et al.*, 1989; Bystrykh *et al.*, 1993a; Hensgens *et al.*, 1993, 1995). The genes coding for the first two enzymes have been cloned and

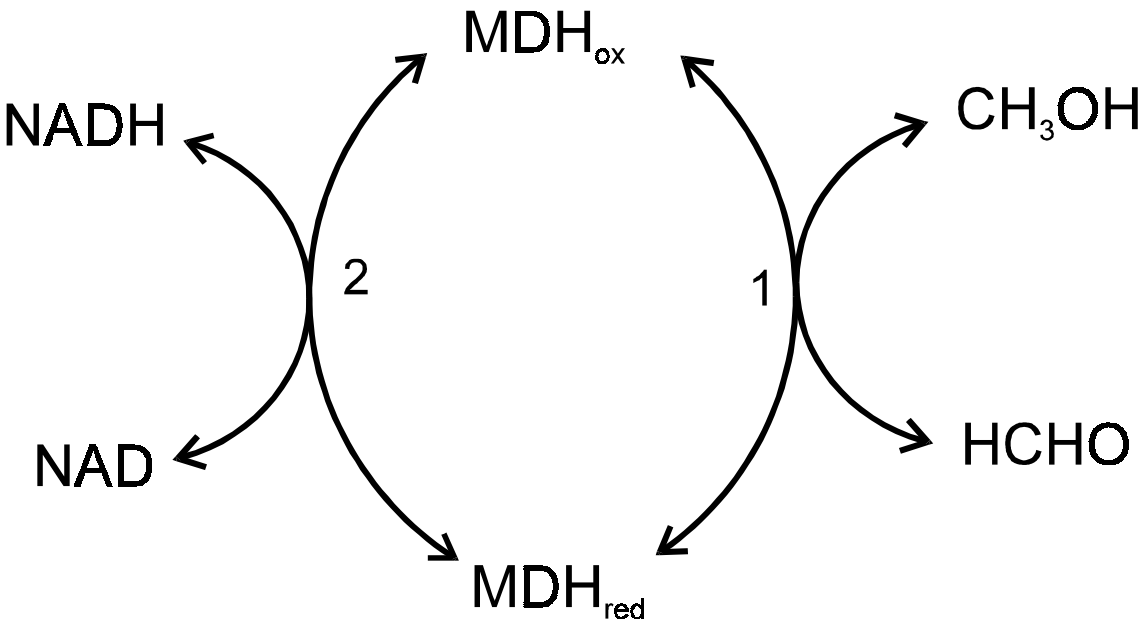

Figure 2. Reaction cycle for MDH of *B. methanolicus*; reaction 2 is facilitated by the activator protein (Arfman *et al.*, 1991, 1997; Chapter 5).

characterized (De Vries *et al.*, 1992; Chapter 4). Classification of the other three enzymes was based on N-terminal amino acid sequence analysis (Fig. 1A). The first three of these enzymes are nicotinoproteins containing a tightly but non-covalently bound NAD(P) per subunit (Bystrykh *et al.*, 1993a; Arfman *et al.*, 1997; Chapter 5). It is not known whether other members of Family III also possess tightly bound NAD(P)(H) molecules. For MDH and *A. methanolica* MNO it has been demonstrated that the bound NAD(P) species act as cofactors; they become reduced when the enzymes oxidize primary alcohols to the respective aldehydes (Bystrykh *et al.*, 1993a; Arfman *et al.*, 1997; Chapter 5). *B. methanolica* MDH requires a second, exogenous NAD for methanol oxidation, serving as a coenzyme and resulting in re-oxidation of the NADH cofactor (Arfman *et al.*, 1997; Chapter 5). *In vitro* the NAD-dependent MDH activity is strongly stimulated by a M_r 50 000 protein from the same organism (Arfman *et al.*, 1991). The re-oxidation step of cofactor NADH is most likely the rate-limiting step and can be stimulated by this activator protein (Fig. 2) (Arfman *et al.*, 1997; Chapter 5), resulting in a 40-fold increase in MDH turnover rate (Arfman *et al.*, 1991).

Little information is available about further structure/function relations in these proteins. Clearly, the unique conserved sequences motifs may represent protein domains with important functions in substrate- or metal-binding, or catalysis. Domain C (position 258 - 290) contains several His residues and is thought to be involved in metal-binding (De Vries *et al.*, 1992; Cabiscol *et al.*, 1994; Bairoch *et al.*, 1996; Tamarit *et al.*, 1997). This remains to be confirmed experimentally, however; the functions of the other conserved regions remain unknown. When the MDH sequence is analyzed for the presence of an NAD-binding domain, the classical dinucleotide-binding fold cannot be identified (De Vries *et al.*, 1992; Chapter 4). The well-known NAD-binding fingerprint, GXGXXG (Wierenga *et al.*, 1986) (Fig. 1A), is absent in MDH and virtually all other Family III enzymes (Chapter 4). This dinucleotide-binding consensus motif in the primary structure results in a βαβ-fold in the secondary structure (Wierenga *et al.*, 1986). The three Gly residues involved allow a tight bending between the α-helices and β-sheets. This enables the dinucleotide to position closely, in the correct conformation, to the protein framework. The coenzyme interacts usually with an D or E residue, forming a hydrogen bond with the 2'-OH of the adenosine ribose moiety. In MDH of *B. methanolicus*, and in most other Family III ADHs, only an imperfect fingerprint ($G^{13}XG^{15}$) is found in the N-terminal part of the protein (Fig. 1A). Obviously, these enzymes contain a strongly modified or novel NAD-binding domain.

The conserved motif A ($VSXGGGSX_2DX_2K$; position 91-103) in Family III ADHs (Table 1; Fig. 1B) displays similarity with FAD-binding domain in certain enzymes (Wierenga and Drenth, 1983; De Hoop *et al.*, 1991). FAD also functions

as a cofactor and remains bound during catalysis, similar to the NAD cofactor in MDH. The enzyme glucose:fructose oxidoreductase of *Zymomonas mobilis* also is a nicotinoprotein, containing a tightly bound NADP(H); its sequence carries a VNSGGGSLMD peptide (Kanagasundaram and Scopes, 1992), very similar to the conserved motif A. No evidence has been reported for a possible role of this sequence in binding of cofactor NADP(H) in the *Z. mobilis* enzyme.

Using site-directed mutagenesis, we modified several amino acid residues in motif A of MDH. The biochemical properties of the purified mutant MDHs are described in this paper. The data provide clear evidence that the G95, S97, D100 and K103 residues of MDH have important roles in binding of NAD(H) cofactor and coenzyme.

Materials and methods

Bacterial strains

Escherichia coli DH5α was cultivated and genetically manipulated as described (Sambrook *et al.*, 1989). Plasmid pMDH (Fig. 3) was constructed using pBluescript KS^+ and the *B. methanolicus* MDH encoding gene, expressed from its own promoter (De Vries *et al.*, 1992).

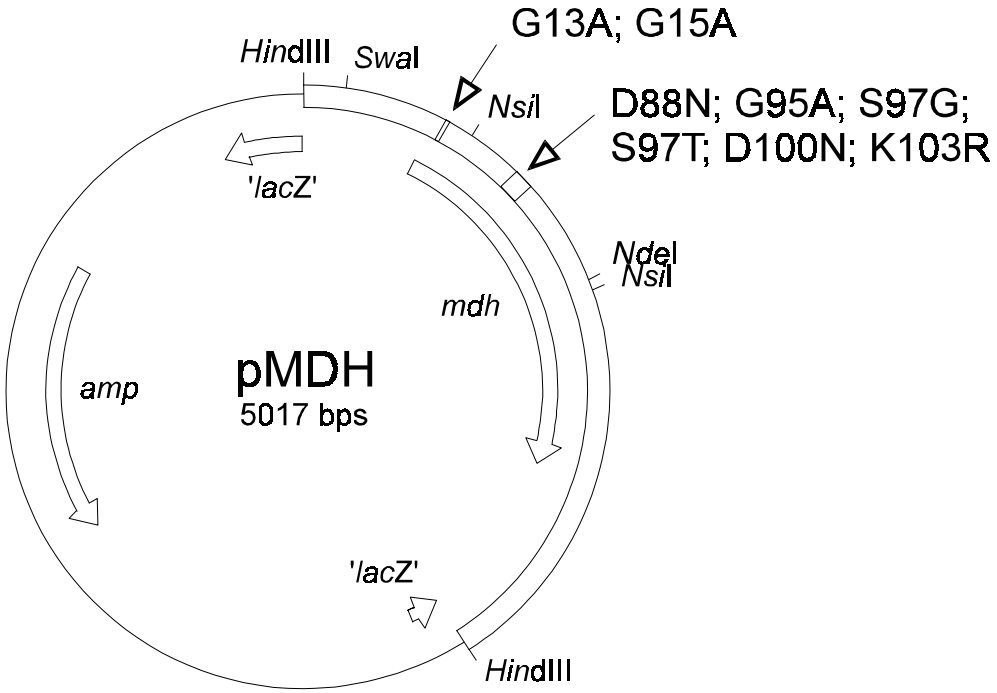

Figure 3. Plasmid pMDH, consisting of pBluescript KS^+ with the MDH-encoding gene of *B. methanolicus*. Arrows indicate the target domains for site-directed mutagenesis.

primer L1	ggtgcagtaaaggaagtagg
primer L2	ggatctagtcgatcagtcg
primer R	cgaatacgtgttatgtaccg

Mutation	Primer	Restriction site
G13A	gcgtaattg**cg**cg**c**ggtgcagtaa V I **A** R G A V	*Bss*H II
G15A	ttggacgag**cc**gc**g**gtaaaggaag G R **A** A V K E	*Sac* II
D88N	gaaaactgt**a**atgc**act****a**g**t**ttctatcg E N C **N** A L V S I	*Spe* I
G95A	ctatcggtg**cc**gg**c**agctctcacg I G **A** G S S H	*Nae* I
S97G	cggtggaggt**g**g**a**tc**c**cacgatacagc G G G **G** S H D T	*Bam*HI
S97T	cggtggagg**g**a**cg**tctcacgatacagc G G G **T** S H D T	*Aat* II
D100N	cggtggagg**ctcgag**tcac**a**atacagc G G G S S H **N** T	*Xho* I
K103R	cgatacag**ccgg**gcaatcgg D T A **R** A I	*Sma* I

Figure 4. Primers for site-directed mutagenesis of MDH of *B. methanolicus.* The mutated nucleotides and amino acids are printed in bold and the resulting restriction sites are underlined.

Secondary structure prediction

The secondary structure of MDH was predicted with the Profile prediction program provided by EMBL Heidelberg (Sander and Schneider, 1991).

Site-directed mutagenesis

Mutations were introduced with the PCR method using VENT-DNA polymerase (New-England Biolabs). With plasmid pMDH as a template for PCR reactions, a first reaction was performed with a mutagenesis primer for the coding strand and a primer 225-345 bp downstream on the template strand. The PCR product obtained was used as primer in a second reaction, together with a primer (primer R; Fig. 4) 415-610 bp upstream on the coding strand. To increase the yield of the PCR, a third reaction was performed with extreme primers and the product of the second PCR as template.

The product of the last PCR step was cut with *Nsi*I and exchanged with the original *Nsi*I fragment of pMDH, resulting in a plasmid which codes for a mutant MDH. Construction of mutants G13A and G15A involved exchange of the *Swa*I - *Nde*I fragment (Fig. 3).

The desired mutants were constructed using the mutagenesis primers listed in Fig. 4; primer L1 was used for mutants G13A and G15A and primer L2 was used for the other six mutants. Figure 4 also lists the new restriction sites introduced by silent mutations, which were used for rapid screening of potential mutants.

All mutations were confirmed by determining the full nucleotide sequences of the mutant genes.

Enzyme assays

All assays were performed at 50°C, using prewarmed buffer solutions. The oxidation or reduction of NAD(H) was followed at 340 nm. The MDH assay contained enzyme, 100 mM Glycine/KOH, pH 9.5, 5 mM $MgSO_4$, 5 mM β-mercaptoethanol and 1 mM NAD; after 3 min pre-incubation the reaction was started with 500 mM methanol (Arfman *et al.*, 1989). The stimulating effect of activator protein was analyzed by subsequently adding saturating amounts of purified activator protein (Kloosterman *et al.*, 1997). The formaldehyde reductase (FoRed) assay contained enzyme, 50 mM potassium phosphate, pH 6.7, and 0.15 mM NADH; after 3 min pre-incubation the reaction was started with 10 mM formaldehyde (Arfman *et al.*, 1989).

Protein purification

Wild type and mutant MDHs were purified as described (De Vries *et al.*, 1992), with some modifications. Overnight cultures of *E. coli* (pMDH) were harvested by centrifugation; cells were disrupted by two passages through a French Pressure cell at 140 MPa. Crude extracts were prepared by centrifugation for 30 min at 40 000 x g. Proteins were partially precipitated by 30 % saturation with ammonium sulphate and incubating for 10 min. Following centrifugation (10 min at 25 000 x g) the supernatant was applied on a phenyl Superose (hydrophobic interaction) column equilibrated with 20 % (w/v) $(NH_4)_2SO_4$ in buffer A (50 mM Tris/HCl, 5 mM $MgSO_4$, 5 mM β-mercaptoethanol, pH 7.5). Proteins were eluted with a gradient of 20 - 0 % (w/v) $(NH_4)_2SO_4$. Active fractions were pooled, desalted on PD-10 columns, and applied on a Mono Q (anion exchange) column; proteins were eluted with a 0 - 1 M KCl gradient in buffer A.

Inactive mutants were purified in the same way; fractions were pooled at the same concentration of the salt gradients as wild type MDH.

Protein determination
Protein concentrations were determined with a Bio-Rad protein determination kit using bovine serum albumine as a standard (Bradford, 1976). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli and Favre (1973). The gel was stained with Coomassie brilliant blue R250.

Kinetic studies
Enzyme kinetics were studied using standard assay conditions and varying substrate concentrations. Data were fitted with Sigma Plot for Windows 2.0 (Jandell Scientific Software) according to the Michaelis-Menten equation. The effect of the activator protein was determined by adding 5 µg purified activator protein (Kloosterman *et al.*, 1997).

Presence of cofactor NAD(H)
The presence of bound NAD(H) cofactor in (mutant) MDH proteins purified from *B. methanolicus* was studied as follows. The spectra of the purified proteins were analyzed for increased absorption at 340 nm, specific for the presence of NADH in wild type MDH (Arfman *et al.*, 1997; Chapter 5). Spectra were recorded on a SLM Aminco DW-2000 spectrophotometer at 50°C using fixed MDH subunit concentrations of 10 µM (6.7 µM for mutant S97T) in 0.1 M Glycine/KOH, pH 9.5, 5 mM $MgSO_4$.

An alternative method involved urea (6 M) denaturation of purified MDH (1-2 mg) (Arfman *et al.*, 1997; Chapter 5); this mixture was boiled for 2 min and separated on a Pharmacia PD-10 column, equilibrated with 10 mM Tris/HCl, pH 8.0, containing 6 M urea (buffer B). The first ml of the salt-fraction, supplemented with 2 ml of buffer B to decrease salinity, was applied on a Mono Q column equilibrated with buffer B, and eluted in a gradient of 0 - 1 M KCl in buffer B. A solution of 10 nmol NADH was treated the same way and served as standard.

Results

Selection of mutants
Analysis of the secondary structure of MDH (Fig. 5), using Profile network prediction Heidelberg (Sander and Schneider, 1991), revealed a repetition of α-helices and β-sheets in the first 140 N-terminal amino acids. The C-terminal part of MDH consists mainly of α-helices and lacks β-sheets. The imperfect NAD-binding fingerprint around G13 and G15 present in the N-terminus of MDH and in many other Family III ADHs (Fig. 1A) does show a βαβ type of fold (Fig. 5). Also the

domain with the conserved motif A shows a similar succession of α-helices and β-sheets; this is not the case for the conserved B and C motifs.

The G13A and G15A mutants were constructed to analyze the possible role of the imperfect NAD-binding fingerprint (GXG). A number of amino acids constituting motif A were selected as targets for mutagenesis, based on the predicted functions for these residues in the known binding motifs. Thus, D88 and D100 may have a direct interaction with the 2'-OH of the adenosine ribose moiety of NAD. G95 may be essential for a proper arrangement of the α-helix and β-sheets, which enables a

```
AA  MTNFFIPPASVIGRGAVKEVGTRLKQIGAKKALIVTDAFLHSTGLSEEVAKNIREAGLDV 60
PHD     βββ    βββ     αααααααααααα    βββββββ    ααα   αααααααααααα    ββ
Rel 9724625721233331799999999937981699957402115615999999999769937

                                      ------A-----
AA  AIFPKAQPDPADTQVHEGVDVFKQENCDALVSIGGGSSHDTAKAIGLVAANGGRINDYQG 120
PHD βββ          αααααααααααααα      βββββββ     αααααααααααααα
Rel 675267989967899999999996399779993999578999999999945683333344

                                                 -------------------B---
AA ´VNSVEKPVVPVVAITTTAGTGSETTSLAVITDSARKVKMPVIDEKITPTVAIVDPELMVK 180
PHD             βββββ           βββββββ         βββββ      βββββ ααααα
Rel 2115799881799974788865331689953344451488716899436552547554 5

    -------------------
AA ´KPAGLTIATGMDALSHAIEAYVAKGATPVTDAFAIQAMKLINEYLPKAVANGEDIEAREA 240
PHD   ααααα     αααααααααααααααααα      αααααααααααααααααααα       ααααααα
Rel 961311233236899999999666323111389999999999999999659988599999

                        ----------------C----------------
AA ´MAYAQYMAGVAFNNGGLGLVHSISHQVGGVYKLQHGICNSVNMPHVCAFNLIAKTERFAH 300
PHD ααααααααααααααααααααααααααααααα   βββ    αααααααααααααααα   αααααααααα
Rel 999999999557635635798863531863425531356765678998652289999999


AA ´IAELLGENVSGLSTAAAAERAIVALERYNKNFGIPSGYAEMGVKEEDIELLAKNAFEDVC 360
PHD ααααα          αααααααααααααααααααα     ααααα         αααααααααααααα
Rel 999955446999917999999999999996179923576248761138999999764832


AA  TQSNPRVATVQDIAQIIKNAL 381
PHD          αααααααααααα
Rel 578999626999999999669
```

Figure 5. Prediction of α-helices (α) and β-sheets (β) in MDH of *B. methanolicus*, according to Profile network prediction Heidelberg (PHD). AA = amino acid sequence, Rel = reliability index of prediction (0-9). The conserved motifs for Family III alcohol dehydrogenases (A, B and C) are indicated; the amino acids modified by site-directed mutagenesis are printed in bold.

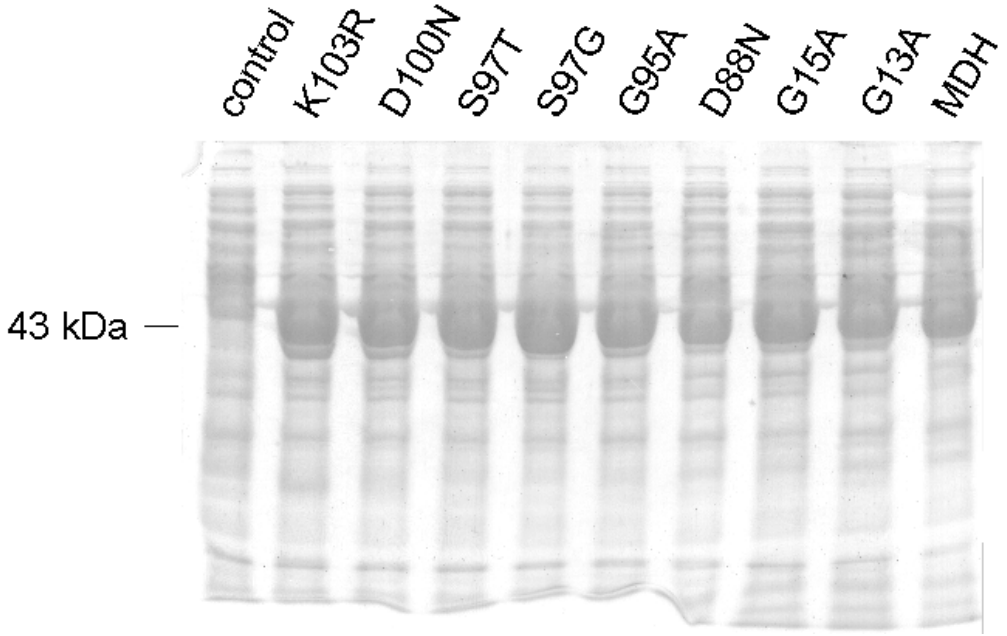

Figure 6. SDS-PAGE of crude extracts of wild type and mutant MDHs. As a control, *E. coli* with pBluescript was treated in the same way. Of every sample 10 µg of protein was loaded on the gel. The size of the dominant band is indicated on the left in kDa.

Table 2. Purification of wild type MDH expressed in *E. coli*.

Sample	Protein mg	Specific activity mU/mg	Total activity mU	Yield %	Purification fold
Crude extract	26.6	20.6	548	100	1
AS-precipitation	13.8	27.9	385	70	1.4
Phenyl Superose	6.5	41.7	270	49	2.0
PD-10	5.3	36.3	192	35	1.7
Mono Q	3.1	55.1	170	31	2.7

close approach of the cofactor/coenzyme to the protein framework and catalytic residues. K103 and S97 were chosen because of their high degree of conservation within the 24 known members of Family III (Fig. 1B). In order to avoid drastic conformational changes, amino acids were replaced by residues of almost the same size, except for the Gly mutants where spherical hindrance was intended. This resulted in the following mutants: D88N, G95A, S97G, S97T, D100N and K103R.

Purification of mutant MDHs

Following expression of mutant MDHs in *E. coli*, SDS-PAGE analysis of crude extracts revealed the dominant MDH band at 43 kDa in all samples (Fig. 6). MDH proteins were purified in three steps. For every mutant the purification fold was about three times, while the yield was approximately 30 %. The purification of wild type MDH provides a representative example (Table 2) (De Vries *et al.*, 1992).

Table 3. Kinetic characteristics of wild type and mutant MDH enzymes expressed in *E. coli*. Calculations are based on Michaelis-Menten kinetics.

	V_{max} (mU/mg)		K_m (mM NAD(H))		Activation[1]
	MDH	FoRed	NAD	NADH	
MDH	390	2100	0.04	<0.01[3]	+
G13A	120	780	0.02	<0.01[3]	+
G15A	430	2500	<0.01[3]	<0.01[3]	+
D88N	350	1700	<0.01[3]	<0.01[3]	+
G95A	220[2]	2300[2]	ND	ND	-
S97G	7780	3000	2.5	0.04	-
S97T	730	2100	0.2	0.02	+
D100N	0	0	ND	ND	-
K103R	0	0	ND	ND	-

ND = not determined. [1] Stimulating effect of activator protein: +: increased activity; -: no effect. [2] MDH and FoRed activities of G95A increased linearly with increasing substrate concentrations; the values given are with 2.5 M methanol and 0.1 M formaldehyde. [3] NAD affinity detection level: 0.01 mM.

Characterization of mutant MDHs

The MDH and the FoRed activities of purified (mutant) MDH proteins were kinetically characterized (Table 3). Mutants G15A and D88N showed minor differences with wild type MDH. These residues thus appear not to play important roles in these activities of MDH. G13A displayed clearly reduced MDH and FoRed activities, most likely due to a decreased protein stability. Mutants D100N and K103R completely lacked both these activities, both in crude extracts and in purified

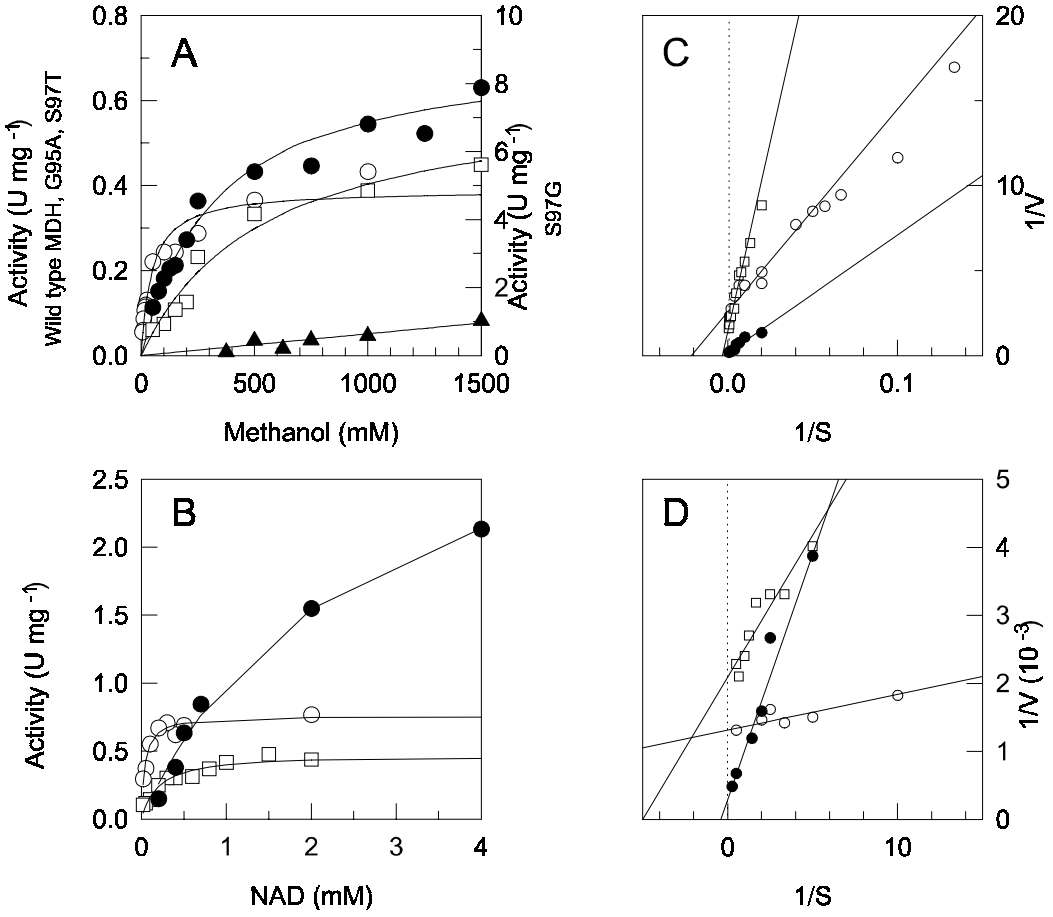

Figure 7. A. MDH activity versus concentration of methanol. B. MDH activity versus concentration of NAD. C. Double reciprocal plots of MDH activity (V) versus methanol concentration (S). D. Double reciprocal plots of MDH activity (V) versus NAD concentration (S). (○) wild type MDH, (▲) G95A, (●) S97G, and (□) S97T.

preparations. These proteins were purified by following the elution profiles of the active enzymes; confirmation of purification was obtained with SDS-PAGE (Fig. 6).

Mutant G95A displayed a clearly diminished affinity for methanol and NAD. When increasing the assay substrate concentrations, the very low MDH activity of G95A increased proportionally (Fig. 7). In contrast to wild type MDH, mutant G95A did not obey Michaelis-Menten kinetics and no kinetic parameters could be estimated in this case. The same was true for the FoRed reaction: increasing amounts of NADH resulted in a linear increase of activity (data not shown) and failure to calculate kinetic parameters.

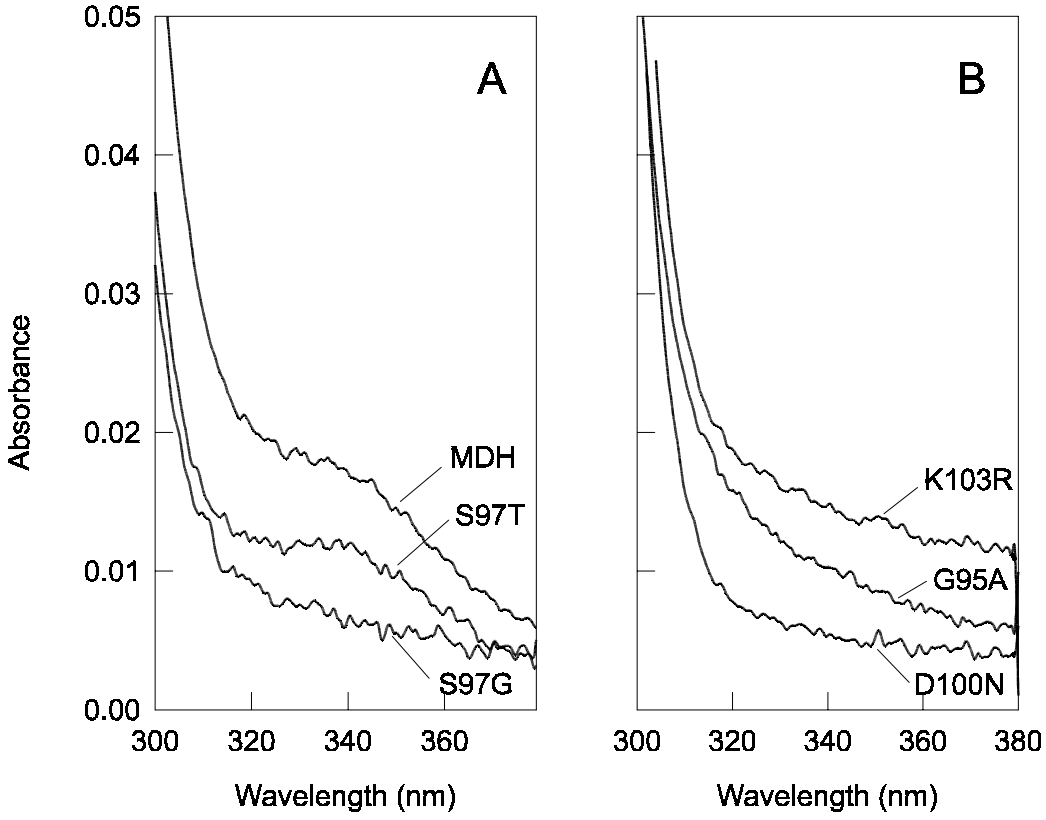

Figure 8. Absorption spectra of purified (mutant) MDH. Spectra were recorded using 10 µM subunits, except for S97T (6.7 µM).

Mutant S97G displayed drastically increased MDH activity. While the V_{max} increased 20 times, the affinity for NAD decreased more than 60 fold (Fig. 7). The mutation S97T had the same effect, albeit less extreme. The V_{max} increased twice and its K_m for NAD was about 5 times higher than in wild type MDH (Table 3). These mutations had no, or less strong, effects on FoRed activity and affinity for NADH.

Mutants D100N and K103R had completely lost both MDH and FoRed activities. SDS-PAGE analysis of crude extracts showed a normal level of expression of these proteins, comparable to the other mutants and wild type (Fig. 6).

The activator protein strongly stimulates MDH (but not FoRed) activity (Arfman *et al.*, 1991). Mutants G95A and S97G were completely insensitive to the stimulating effect of the activator protein on MDH activity. The inactive mutants D100N and K103R remained inactive after addition of activator protein. All other active MDHs, including wild type, were activated at least 3 to 4 times when a saturating amount of activator protein was added.

Presence of cofactor

Two methods were used to establish the presence of bound NAD(H) cofactor in (mutant) MDHs, namely analysis of the absorbance spectra of the purified proteins and of the extracted cofactor fractions of the denatured proteins. The absorbance spectra of wild type MDH and five mutants are shown in Fig. 8. MDH and S97T clearly show a shoulder at 340 nm, while G95A, S97G, D100N and K103R completely lack this shoulder. These four mutant MDHs thus appear to have lost the NADH cofactor.

Further confirmation was sought by analyzing the cofactor contents of urea extracts of MDH proteins, using Mono Q anion exchange chromatography (Fig. 9). The elution profiles of the cofactor fractions of wild type MDH (2 mg) and mutant S97T (1.6 mg) clearly showed absorbance peaks at 260 nm, corresponding to NADH. A similar amount of S97G protein (1.5 mg) was used for urea treatment, but in this case the A260 peak was absent in the elution profile. The amounts of G95A, D100N and K103R used for denaturation were 1.2 mg, 1 mg, and 1.1 mg, respectively. In these cases the elution profiles only showed minor or no peaks. The data thus clearly indicate that only S97T still binds its cofactor, while mutants G95A, S97G, D100N and K103R lack bound NADH, or contain only minor amounts.

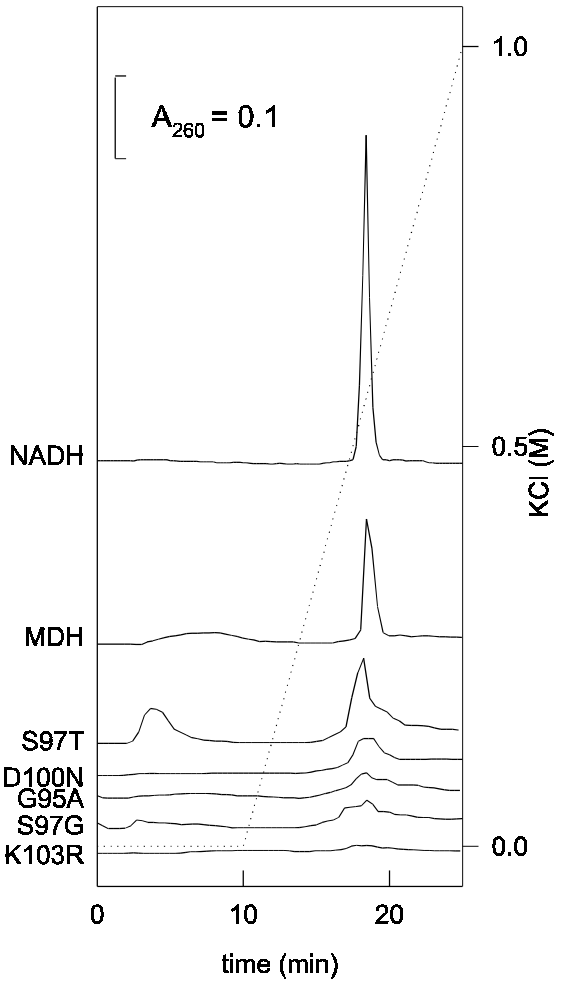

Figure 9. Elution profiles of cofactor fractions on a Mono Q anion exchange column. Amount of protein used for denaturation: MDH (2 mg), G95A (1.2 mg), S97G (1.5 mg), S97T (1.6 mg), D100N (1 mg) and K103R (1.1 mg). Elution profile of NADH (10 nmol) is used as reference. The dotted line represents the concentration KCl in the buffer.

Discussion

The genes encoding mutant MDHs were completely sequenced, confirming that only the intended, single amino acid substitutions indeed had occurred. Any differences in MDH properties therefore could be attributed to primary or secondary

effects of the single amino acid modifications. The data thus show that G13 and G15 in the N-terminal part of the protein are not involved in binding of cofactor or coenzyme NADH. These mutant enzymes showed normal characteristics, albeit that mutant G13A displayed decreased MDH and FoRed V_{max} values. This is most likely due to a slightly reduced protein stability. Also mutant D88N displayed no significant differences with wild type MDH.

Mutants D100N and K103R had completely lost MDH and FoRed activities, both in crude extracts and in purified protein preparations. These proteins displayed a normal mobility on SDS-PAGE and there were no indications for loss of protein stability. Both the absorption spectra and cofactor elution profiles indicated that these mutants had lost the NADH cofactors. The FoRed reaction is not stimulated by the activator protein; the NADH cofactor thus does not appear to have a role in formaldehyde reduction (Arfman *et al.*, 1991). The zero activity in these mutants therefore is not solely due to their loss of ability to bind the cofactor. Most likely these mutants are affected in a property important for both the MDH and the FoRed reactions, for instance the temporary binding of coenzyme NAD(H). This would indicate that the NAD(H) cofactor- and coenzyme-binding sites in MDH are either sterically very close or even have overlap with each other. Mechanistically this would make sense, allowing transfer of electrons from the bound cofactor to the coenzyme. The precise functions of the D100 and K103 residues remain unknown. In NAD(H)-binding sites of other enzymes with the GXGXXG fingerprint motif, an Asp residue is directly involved in binding of the cofactor or coenzyme, but this residue is at a position clearly different from that of D100 in MDH (Compare Fig. 1A, B) (Wierenga *et al.*, 1986; Brandon and Tooze, 1991). Also Lys is a common conserved residue in these sites (Brandon and Tooze, 1991), but its role is not known.

Mutants G95A and S97G were most strongly affected in the MDH reaction and were both lacking cofactor NADH. These two amino acids therefore appear to be involved in the binding of the NADH cofactor. Interestingly, S97T still binds cofactor and is stimulatated by the activator protein, whereas S97G is free of cofactor NAD(H). It may therefore be speculated that S97 is forming a hydrogen bond with cofactor NAD(H), analogous to the role of the Asp residue in the known binding consensi for NAD(P)(H). T97, with an extra methyl group, may still be able to form a hydrogen bond; this will not be possible in mutant S97G. G95 may be involved in positioning of S97 in the active site, to allow a close interaction with cofactor NAD(H).

Both the MDH and FoRed reactions of mutant G95A no longer obeyed Michaelis-Menten kinetics. The S97G,T mutants still showed Michaelis-Menten kinetics but their affinities for coenzyme NAD had decreased (60 and 5 fold,

respectively). The binding sites for cofactor and coenzyme are most likely closely linked. Loss of cofactor in S97G may therefore seriously affect binding of the coenzyme. Wild type MDH displays a ping-pong type of mechanism, with the cofactor functioning as temporary electron sink. It is likely that loss of the cofactor has a strong effect on enzyme kinetics. The kinetic mechanisms of mutants G95A and S97G,T remain to be studied, however.

The extremely high coenzyme NAD-dependent MDH activity of S97G is very interesting. It is hard to imagine how a simple point mutation can have such an enormously stimulating effect. Natural selection might have resulted in appearance of this enzyme if the high activity would have also an *in vivo* positive effect. Such a highly active MDH thus may be detrimental for *B. methanolicus*, for instance causing accumulation of toxic levels of formaldehyde. Further *in vivo* studies with mutant MDHs have to await the development of methods for genetic manipulation of *B. methanolicus* strains.

More work is needed to identify the complete binding domains of the NAD(H) cofactor and coenzyme in MDH. Analogous to observations with other binding domains for NAD(H) and FAD, it is likely that residues in different parts of the amino acid sequence, but all contributing to the active site, are involved. Also interactions between cofactor and amide groups of the backbone have been suggested (Baker *et al.*, 1992). This makes it virtually impossible to define the complete cofactor domain, without crystal structures.

Several residues of the conserved motif in domain A of Family III ADHs thus are indeed involved in binding of cofactor NAD(H). It remains to be studied whether tight binding of NAD(P)(H) cofactors is a lot more widespread than thus far reported among Family III ADHs.

Acknowledgements

Thanks are due to R.K. Wierenga (Heidelberg) and P. Terpstra (Groningen) for valuable suggestions for targets of site-directed mutagenesis. We are indebted to A. Boorsma for technical assistance.

References

References are listed on pages 127 - 136.